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Letter to the Editor

Measurement of Human Antibodies to Type III Group B *Streptococcus*

We disagree with the conclusions reached by Bhushan et al. (5) in their study comparing different methods of coating enzyme-linked immunosorbent assay (ELISA) plates for estimation of group B *Streptococcus* type III (GBS III) polysaccharide-specific antibody concentrations in human serum. We believe that interpretation of the data as suggested in their paper could lead to serious errors in the use of serum antibody concentrations as surrogates of vaccine efficacy. We propose four alternate conclusions that are supported by the data given by Bhushan et al. (5).

(i) ELISAs that used free GBS III polysaccharide or polysaccharide mixed with methylated human serum albumin (mHSA) as a coating antigen failed to detect all of the antibodies reactive with type III polysaccharide in a quantitative precipitin assay. Bhushan et al. found that ELISA methods using GBS III polysaccharide alone or type III polysaccharide mixed with mHSA as coating antigens measured substantially lower concentrations of specific immunoglobulin G (IgG) in a reference serum than did methods using polysaccharide covalently conjugated to HSA or to biotin. However, the specific IgG concentration detected by the conjugated polysaccharide methods in ELISAs agreed very closely with the concentration of specific antibodies in the reference serum (standard human reference serum III [SHRS III]) determined by quantitative precipitin analysis with the purified type III polysaccharide as antigen, while the ELISA methods using unconjugated polysaccharide underestimated the antibody concentration by more than 50%. The data provided in the paper are inadequate to permit the assessment of the relative sensitivities of the free versus conjugated polysaccharide methods, but experiments in our laboratory have shown that assays using free polysaccharide as the coating antigen are less sensitive than those using polysaccharide conjugated to HSA (III-HSA) (6).

(ii) Antibody measurements in the III-HSA assay are highly correlated with antibody measurements in the RABA. The radioactive antigen binding assay (RABA) is an assay in which soluble polysaccharide (unencumbered by attachment to a plastic surface) is allowed to interact in solution with antibodies; the antibody concentration is determined by quantifying the amount of polysaccharide complexed to specific antibodies. The RABA is the gold standard assay because it is the only immunoassay shown to give results that correlate with neonatal susceptibility to GBS III infection (1–3).

To test the validity of the unconjugated polysaccharide ELISA, III-specific IgG in 16 serum specimens from healthy adults was measured by ELISA independently in the laboratory of Bhushan et al. at the Food and Drug Administration (FDA) and by RABA in the laboratory of one of us (C.J.B.). Both the FDA and the Baylor laboratories also measured specific IgG concentrations by the III-HSA ELISA method. Antibody concentrations determined by the RABA and the III-HSA ELISA (irrespective of the laboratory in which the III-HSA ELISA was performed) were in better agreement than those determined by RABA and the free polysaccharide plus mHSA method (unpublished data). These results support the previously published excellent correlation ($r = 0.92$) between antibody concentrations determined by the III-HSA ELISA and by the RABA (6). Of particular concern is the overestimation of specific antibodies by the free polysaccharide plus mHSA method in the eight serum samples that contained <1.0 μg of specific antibodies per ml according to RABA. In those eight samples, the range of specific IgG concentrations determined by III-HSA ELISA was <0.1 to 0.5 μg/ml at the FDA and <0.05 to 0.47 μg/ml at Baylor. By contrast, the free polysaccharide plus mHSA method measured >1.0 μg of specific IgG per ml (range, 1.0 to 4.2 μg/ml) in each of the samples. Overestimates by the free polysaccharide plus mHSA method are of serious concern because Bhushan et al. propose to use this ELISA to establish the minimum level of maternal antibody that is protective against neonatal infection. Overestimation of specific antibody levels would yield a falsely elevated value for the minimum protective level in such studies.

(iii) The III-HSA ELISA measures antibodies that cross-react with PN-14 polysaccharide but that also bind to GBS III polysaccharide. Bhushan et al. suggest that conjugation of the type III polysaccharide to a protein alters the antigenic specificity of the polysaccharide. Because the type III ELISA methods using conjugated polysaccharide also detected antibodies cross-reactive with the structurally related type 14 pneumococcal (PN-14) polysaccharide, the investigators conclude that the conjugated polysaccharide ELISAs are less specific than assays using free polysaccharide. Antibody cross-reactions between GBS III and PN-14 polysaccharides have been recognized for many years (4, 6–8). We have reported that in some subjects immunization with the purified type III polysaccharide evokes antibodies that cross-react with PN-14 polysaccharide (9). Of importance is that these are truly cross-reacting antibody populations: the GBS III-HSA ELISA measured no increase in specific antibodies in the postimmunization sera of four patients who responded to vaccination with PN-14 polysaccharide (6). Thus, the III-HSA ELISA detects antibodies that bind to type III polysaccharide and cross-react with PN-14, but it does not detect all antibodies that recognize PN-14. The lower sensitivity of the free polysaccharide method accounts for the inability of this method to detect lower-affinity antibodies such as those that cross-react with PN-14 polysaccharide.

(iv) The III-HSA ELISA, but not the free polysaccharide ELISA, has been shown to correlate directly with opsonic activity of serum against GBS III. In addition to the problem of assay sensitivity, it is critical to determine which assay system gives results that most closely reflect the concentration of functionally active and protective antibodies. Specific IgG concentrations measured by the III-HSA method have been shown to correlate with opsonophagocytic killing activity of the antisera to GBS III in sera from subjects immunized either with type III polysaccharide or with polysaccharide conjugated to tetanus toxoid (10). No such relationship with functional activity has been demonstrated for antibody detection by the free polysaccharide method.

On the basis of these data and the results presented in the article by Bhushan et al. (5), neither the free polysaccharide method nor the polysaccharide plus mHSA ELISA method can be considered a valid technique for measurement of naturally acquired GBS III polysaccharide-specific IgG in human serum.

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Authors' Reply

Methods for estimation of human antibodies to the type III polysaccharide (PS) of group B streptococcus (GBS) should specifically measure antibodies to the type III PS. Studies by Bhushan et al. (4) were initiated to compare different ELISA antigens for their sensitivity and specificity for measurement of immunoglobulin G (IgG) anti-GBS PS antibodies. We found the enzyme-linked immunosorbent assay (ELISA) described by Guttormsen et al. (7) to be sensitive but it lacked the needed specificity for estimation of GBS type III PS antibodies as shown in Fig. 1A of Guttormsen et al. (7) and Fig. 5 of Bhushan et al. (4), where binding to the pneumococcal type 14 (PN-14) PS was evident. The lack of specificity was overcome by working out conditions for attachment of the free GBS type III PS to the ELISA plate.

While there is no perfect method for estimation of these antibodies, in our hands, free GBS type III PS bound directly to the plate or mixed with methylated human serum albumin (mHSA) measured most specifically GBS type III antibodies. The sensitivity was sufficient to measure at least 0.05 μg of IgG antibody per ml, similar to that reported by Guttormsen et al. for their assay (7).

We have addressed the four alternative conclusions proposed by Kasper and coworkers. We disagree with their conclusions proposed in response to our paper (4).

(i) The question is about antibody specificity, not use of quantitative precipitation per se. We have compared antibody concentrations estimated using four different PS preparations in reference sera, sera from immunized and from nonimmunized adults. We found that apparent assay sensitivity was dependent on the serum used. When reference serum 19, a pool of sera from adults immunized with a GBS tetravalent PS vaccine containing low levels of PN-14 antibody, was used at an antibody concentration of 40 to 80 ng/ml, antibodies bound equally to both free and conjugated GBS type III antigen preparations (4). When the SHRS-III reference serum, a serum pool prepared from five individuals receiving GBS type III-tetanus toxoid conjugate, was used at the same antibody concentration, marked differences were seen between antibody binding to conjugated PS preparations and to free PS or PS mixed with mHSA. Antibody concentrations calculated in micrograms per milliliter for two GBS hyperimmune immune globulin intravenous (IGIV) preparations (004 and 006) were also comparable with all four antigen preparations (4, 6). Thus, when the postvaccination serum contained mostly GBS type III-specific PS antibodies, there was little difference in antibody binding among the four antigen preparations, but when the serum also contained antibodies to the PN-14 PS, then marked differences were seen in measured antibody concentrations among the different antigen preparations. Analyzing SHRS-III, when PS alone or PS mixed with mHSA were used as ELISA coating antigens, the GBS type III antibody concentration was less than half (33 μg/ml) of that estimated when PS conjugated to biotin or HSA (81 to 83 μg/ml) was used as coating antigen. The large differences in antibody concentrations estimated in SHRS-III are because SHRS-III also contains 41.0 μg of PN-14 PS antibody per ml.

(ii) Kasper and coworkers correlate antibody concentrations determined by GBS III-HSA conjugate ELISA with a radioactive antigen binding assay (RABA). The GBS PSs induce IgG, IgA, and IgM antibodies (1, 2, 7). Our purpose was to specifically measure those antibodies that will potentially cross the placenta to protect the neonate. We therefore made no comparisons with RABA. Guttormsen et al. (7) showed that GBS III RABA did not measure antibody concentrations <1 μg/ml because of the limitation in preparing labeled PS of sufficiently high specific activity. RABA also does not distinguish among immunoglobulin isotypes or subclasses (3, 7) and measures only precipitable antibody. Protection against GBS disease is mediated by opsonsophagocytosis and is the functional correlate of protection.

(iii) Since antibodies to PN-14 PS are not protective against GBS disease (8), we think an assay that specifically measures antibody to GBS type III PS, and is not sensitive to binding of PN-14 PS antibodies, would be appropriate to determine maternal anti-PS antibody concentrations necessary for protection against GBS disease. Earlier studies done by Kasper and coworkers (8) showed that rabbit antiserum to PN-14 did not react with GBS type III native PS in agar gel diffusion but reacted strongly with partially desialyated GBS core PS. They immunized a group of adult volunteers with GBS type III native PS, GBS core PS, or polyvalent pneumococcal PS vaccine. Sera from immunized volunteers receiving the GBS type III vaccine were highly opsonic, while only 1 of 12 serum samples from volunteers receiving GBS core PS or pneumococcal vaccine had an opsonic response to GBS type III. They also demonstrated that development of opsonic antibody and natural immunity to GBS was correlated with antibody to the native GBS type III PS rather than antibody to the core PS.

Thus, if antibody to PN-14 does not protect against GBS disease, why use an assay that is also sensitive to binding of antibodies to the PN-14 PS?

We are concerned that GBS III-HSA ELISA will overestimate antibody concentrations if the serum contains PN-14 PS antibody and will underestimate GBS III-specific antibodies because the change in conformation of the PS (after conjugation) that results in a PN-14-reactive epitope replaces an alternative GBS type III-specific epitope.

We have also compared four different GBS Ia PS preparations as coating antigens in ELISA to determine GBS type Ia PS-specific antibodies (free PS, PS mixed with mHSA, PS conjugated to biotin, or PS conjugated to HSA) (5). These PS preparations were used to evaluate GBS Ia antibody in sera from recipients of a GBS tetravalent PS (Ia, Ib, II, or III) vaccine, in sera from women receiving GBS type Ia-conjugated PS vaccine, and in sera from nonimmunized healthy women of childbearing age. Unlike the GBS type III ELISA, GBS Ia PS antibody concentrations estimated by all four PS antigen preparations were similar (5).

(iv) IgG concentrations measured by using free PS or PS mixed with mHSA correlate well with opsonic activity. The correlation coefficients of ELISA titers versus opsonic titers were 0.90 by using PS mixed with mHSA as the coating antigen and 0.60 by using PS conjugated to HSA for sera from nonimmunized women. We have also looked at GBS type III postvaccination sera from adults, and the correlation coefficients for antibody concentrations versus opsonic titer for PS mixed with mHSA and for PS conjugated to HSA were 0.63 and 0.52, respectively (5). Thus, an ELISA using free GBS type III PS or PS mixed with mHSA correlates better with functional activity.

In conclusion, conjugate vaccines are currently under investigation for protection of women and neonates against GBS disease (9). There is always a potential problem when antibody responses induced by a conjugate vaccine (for example GBS type III conjugated to tetanus toxoid) are measured by using antigens similar to the vaccine (GBS III-HSA). We found that the GBS III-HSA ELISA overestimated the SHRS-III response because it also measured antibodies to a PN-14 PS reactive epitope created following conjugation. Since it is unlikely that classical randomized efficacy trials will be possible in the United States because of the highly successful implementation of the Centers for Disease Control and Prevention prophylaxis guidelines (10), it is important to use antibody assays that specifically measure IgG anti-GBS PS antibodies.

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